Rheumatology 2002;41:223–227

Paediatric Rheumatology/Series Editor: P. Woo

Tumour necrosis factor α G→A −238 and G→A −308 polymorphisms in juvenile idiopathic arthritis

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Abstract

Objectives. To study G→A −238 and G→A −308 polymorphisms in the promoter region of the tumour necrosis factor (TNF) α gene in patients with juvenile idiopathic arthritis (JIA). We analysed whether there were any associations between these polymorphisms and the type of JIA and/or the clinical course of the disease in two populations.

Methods. The first group consisted of 51 Turkish JIA patients and the second consisted of 159 JIA patients from the Czech Republic. Healthy individuals (93 and 100) from each country served as controls. Subgroups of JIA were defined according to the Durban criteria. The course of the disease was defined on the basis of the physician's global evaluation of disease activity, the swollen and tender joint count and the erythrocyte sedimentation rate.

Results. In both JIA cohorts, the distribution of genotypes was not significantly different among the types of JIA. The G→A −238 polymorphism did not have an effect on the patients' outcome in either group. The G→A −308 polymorphism was significantly associated with a poor outcome in the Turkish group ($P = 0.005$) but there was no association in the Czech patients. Some features of JIA in Turkish patients differed from those in Czech patients.

Conclusions. Genetic differences may accompany the phenotypic differences found in the Turkish group. Although larger numbers of patients are clearly needed to verify this, we suggest that the G→A −308 polymorphism may be operative in defining disease outcome in selected groups.

KEY WORDS: Juvenile idiopathic arthritis, TNF-α polymorphism.

Juvenile idiopathic arthritis (JIA) is the most common rheumatological disease of childhood. Certain genetic factors acting in concert are believed to predispose the host to the development of JIA. Linkage studies and association studies have been carried out to delineate the factors involved in various rheumatological diseases. Association studies test whether a certain allele of a given gene occurs more frequently in affected individuals than in unaffected individuals from the same population. In JIA, the best-defined genetic associations have been with HLA [1]. In recent years, a number of cytokine associations have been related to disease activity in JIA. Crawley *et al*. [2] showed that a certain IL-10 haplotype (ATA) was associated with extended oligoarthritis. This polymorphism was in turn associated with lower production of interleukin (IL)-10, an anti-inflammatory cytokine. McDowell *et al*. [3] showed that an IL-1α polymorphism was correlated with ocular complications in oligoarticular JIA. A number of cytokine gene associations have also been associated with the severity of adult rheumatoid arthritis.

Tumour necrosis factor (TNF) α is a cytokine that plays an important role in acute inflammation. In oligoarticular and polyarticular JIA, plasma levels of TNF-α have been correlated with disease activity [4]. A number of polymorphisms in the TNF-α gene (*TNFA*) promoter have been defined. Some of these polymorphisms may affect the level of TNF-α expression; for example patients with TNF-α G→A heterozygosity at position

Submitted 19 March 2001; revised version accepted 6 July 2001.

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−308 have increased TNF-α production [5–7]. Wilson *et al.* [7] have suggested that the polymorphism at this position has direct effects on the regulation of the *TNF* gene and may be responsible for greater severity of certain infectious diseases. Despite data indicating that this polymorphism in the promoter region up-regulates *TNF* transcription, the functional importance of this putative allele remains controversial [6]. More specific investigations of multiple genetic markers at this locus are needed if we are to understand whether this polymorphism is functional *per se* or whether it is in linkage disequilibrium with another polymorphism [6].

TNF-α polymorphisms have been associated with the course of adult rheumatoid arthritis, and genetic variation within the TNF-α locus seems to be important in disease susceptibility and outcome [8, 9]. Allelic variation at the TNF-α locus has been associated with the course of disease in a number of rheumatological conditions. A G→A point mutation in the promoter region has been associated with a number of rheumatological diseases, including systemic lupus erythematosus (SLE) [10, 11]. Rood *et al.* [11] have shown that both the *TNF*-308 A/A and the 308 G/A genotype occurred at a higher frequency higher in SLE patients than in controls. Their study also showed that the *TNF*-308A allele was a susceptibility factor for SLE, and this effect was independent of HLA-DR3.

We investigated whether the G→A −238 and G→A −308 polymorphisms in the promoter region of the *TNF*α gene were associated with the type of JIA and/or the clinical course of the disease.

Patients and methods

JIA was diagnosed according to the Durban classification criteria [12]. Thus, patients with oligoarthritis were subgrouped as having a persistent or extended form of the disease. Patients with polyarthritis were divided into those with and without rheumatoid factor. Enthesitis-related arthritis and psoriatic arthritis were defined as indicated in the Durban criteria [12].

The patients were from two centres. The first group consisted of 51 children diagnosed at the department of Paediatric Rheumatology and Nephrology, Hacettepe University, Turkey, and the second group consisted of 159 patients from the Institute of Rheumatology and the Department of Paediatrics, Charles University, Prague, Czech Republic. Patients who did not fit into a category of the Durban criteria were excluded. We recruited 93 and 100 healthy controls for the Turkish and Czech groups respectively. The patient groups were evaluated separately because the distribution of the genotypes was expected to be different.

DNA was isolated from blood samples drawn at the time of sampling for routine laboratory tests. Ethical consent was obtained from all families.

DNA analysis

The 238 G→A and 308 G→A polymorphisms were analysed in DNA extracted from whole blood. Genotyping for 238 G→A was performed with a polymerase chain reaction (PCR) fragment amplified with the forward primer 5′ AAA CAG ACC ACA GAC CTG GTC 3′ and the reverse primer 5′ CTC ACA CTC CCC ATC CTC CCG GAT C 3′. For the 308 G→A polymorphism, we used the forward primer 5′ GAG GCA ATA GGT TTT GAG GGC CAT 3′ and the reverse primer 5′ GGG ACA CAC AAG CAT CAA G 3′. PCR was performed in a final volume of 25 μl containing 2.5 μl *Taq* polymerase buffer, 1 μl of each primer, 2 μl dNTP, 0.2 μl magnesium (0.4 μl for the 308 G/A polymorphism), 1 μl dimethyl sulphoxide and 0.2 μl *Taq* polymerase. The PCR conditions were as follows: denaturation for 94°C at 3 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 1 min and extension at 72°C for 2 min, followed by final extension at 72°C for 5 min. PCR products were electrophoresed through 2% agarose gel.

The amplification products of the 238 G→A polymorphism were digested with the restriction enzyme *Bam*HI for 3.5 h at 37°C. The amplification products of the 308 G→A polymorphism were digested with *Nco*I for 3.5 h at 37°C. The products were electrophoresed through 2% agarose gel.

At the time of diagnosis, joint findings were recorded for each patient and the patient's doctor was asked to assess disease activity on a scale of 1–10. Complete blood counts, erythrocyte sedimentation rate (ESR), anti-nuclear antibody (ANA) and rheumatoid factor were evaluated for each patient. The number of swollen and tender joints was counted and evaluated at the end of follow-up [13].

The patients were defined as having a good clinical course if they had a decrease of $\geqslant 50\%$ each in the physician's global evaluation of disease activity, the number of swollen and tender joints and ESR. The patient was defined as having a poor prognosis if he or she did not meet these criteria.

All patients were evaluated by an ophthalmologist at the beginning of the study and every 3–12 months thereafter, depending on the disease subgroup and ANA positivity.

Statistical analysis

The allele frequencies were determined in the cases and controls and were compared with the values predicted by the Hardy–Weinberg equilibrium by means of the χ^2 test. Odds ratios were calculated as a measure of the association of the *TNF* genotype with the phenotype. For each odds ratio, two-tailed *P* values and 95% confidence intervals were calculated. Leuvene's two-tailed *t*-test was used to compare the means of variables.

Results

Turkish group

The mean age of the patients was 7.93 ± 4.35 yr (range 1.5–16). There was a total of 51 patients. The

female : male ratio was 1.12 : 1. The mean follow-up period was 4.15 ± 3.67 yr (range 1.0–15).

ANA was present in 22.7% of the persistent oligoarthritis patients. Rheumatoid factor was present in 10% of the polyarticular patients. ESR at the time of diagnosis was 67.65 ± 28.10 mm/h (range 10–150). All patients with oligoarthritis (Table 1) were treated with non-steroidal anti-inflammatory drugs (NSAIDs) plus intra-articular steroid injections when deemed necessary. All patients with polyarthritis and extended oligoarthritis received methotrexate (MTX) at 12.5 mg/m^2/week orally or subcutaneously, plus NSAIDs and intra-articular steroids. Twenty-five per cent received short courses of corticosteroids by the oral or intravenous route. All patients with systemic arthritis received oral corticosteroids plus MTX and NSAIDs. All patients with enthesitis-related arthritis received Salazopyrin (sulphasalazine) plus NSAIDs.

Allele frequency distributions of the G→A −238 and G→A −308 TNF-α polymorphisms in the patients were not significantly different from those of the controls (Table 2). The distributions of allele frequencies were not significantly different among the types of JIA (all $P > 0.05$). However, the frequency of the G allele was higher in persistent oligoarticular patients, although the difference did not reach significance (86.7 *vs* 57.1%).

The G→A −238 polymorphism did not have an effect on the patients' outcome, whereas the G→A −308 polymorphism was significantly associated with a poor outcome ($P = 0.005$) (Fig. 1). A total of 13 patients carried the A allele at this position and 11 had a poor disease outcome.

TABLE 1. Characteristics of the Turkish patients

Type of JIA patients	Number of patients	Female : male ratio
Oligoarticular	22	15 : 7
Oligo-persistent	12	8 : 4
Extended oligo	7	6 : 1
Enthesitis-related	3	1 : 2
Polyarticular	20	10 : 10
Systemic	9	2 : 7
Total	51	27 : 24

TABLE 2. TNF polymorphisms according to type of JIA in the Turkish group

	Polymorphism			
	G→A −238		G→A −308	
	G allele % (*n*)	A allele % (*n*)	G allele % (*n*)	A allele % (*n*)
JIA patients (total)	78.4 (40)	21.6 (11)	74.6 (38)	25.4 (13)
Oligo-persistent	86.7 (13)	13.3 (2)	86.7 (13)	13.3 (2)
Extended oligo	57.1 (4)	42.9 (3)	85.7 (6)	14.3 (1)
Polyarticular	85.0 (17)	15.0 (3)	60.0 (12)	40.0 (8)
Systemic	66.7 (6)	33.3 (3)	77.8 (7)	22.2 (2)
Controls	73.1 (68)	26.9 (25)	68.8 (64)	31.2 (29)

Czech group

The mean age of the patients was 8.96 ± 4.62 yr (range 1–16). There was a total of 159 patients, of whom 60.5% were girls. The female : male ratio was 1.49 : 1. The mean follow-up period ranged from 4.0 to 19 yr.

The subgroup with enthesitis-related JIA, which constituted about one-third of the patients, was very large compared with that in the Turkish group (Table 3). ANA was found in 70.4% of the persistent oligoarthritis patients and 37.8% of the whole group. Again, the allele frequency distributions for the G→A −238 and G→A −308 TNF-α polymorphisms were not significantly different from those of the controls. The distributions of allele frequencies did not differ significantly among the types of JIA (all $P > 0.05$) (Table 3). The two polymorphisms studied did not

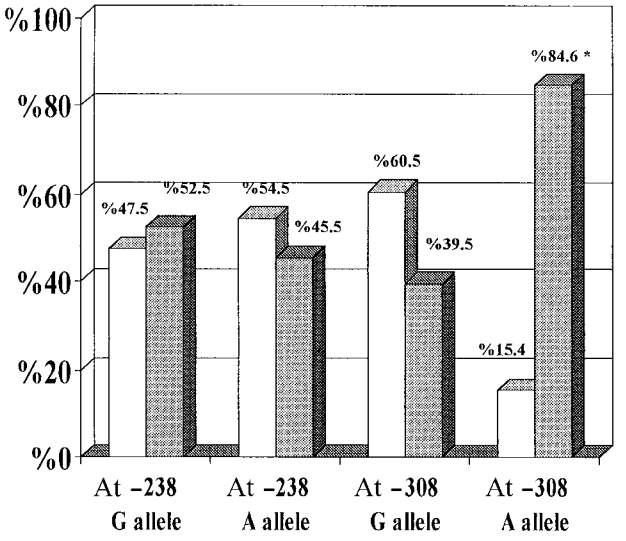

FIG. 1. The association of the clinical course with the two TNF-α polymorphisms in the Turkish patients. *$P < 0.05$. Good prognosis, clear columns; bad prognosis, shaded columns.

TABLE 3. TNF polymorphisms according to type of JIA in the Czech group

	Polymorphism			
	G→A −238		G→A −308	
	G allele % (*n*)	A allele % (*n*)	G allele % (*n*)	A allele % (*n*)
JIA patients (total)	76.7 (122)	23.3 (37)	73.0 (116)	27.0 (43)
Oligo-persistent	74.1 (20)	25.9 (7)	66.7 (18)	33.3 (9)
Extended oligo	84.2 (16)	15.8 (3)	73.7 (14)	26.3 (5)
Enthesitis-related	74.6 (44)	25.4 (15)	72.9 (43)	27.1 (16)
Polyarticular	75.6 (31)	24.4 (10)	75.6 (31)	24.4 (10)
Systemic	84.6 (11)	15.4 (2)	76.9 (10)	23.1 (3)
Controls	83 (83)	17 (17)	84.5 (84.5)	15.5 (15.5)

correlate with the severity of the disease, analysed as described above.

Discussion

The Turkish group showed some notable phenotypic differences compared with data published previously and with the Czech group. In data based from western Europe and the USA, the female:male ratio is around 2 : 1 [1]. In the Turkish group males increased, with a female : male ratio of only 1.12 : 1. The increased proportion of males has been commented on previously by other studies from Turkey [14, 15]. The proportion of females in the Czech group was also somewhat lower than in the western data; this may be attributed to the large group of patients with enthesitis-related JIA, most of whom were boys. Another difference is ANA positivity. ANA was present in 65–85% of the oligoarthritis-uveitis patients in North America, but had a much lower frequency in the Turkish group in the present study. Again, the lower frequency of ANA positive oligoarthritis patients in the Turkish group has been noted previously [14]. On the other hand, in the Czech group the frequency of ANA-positive patients was compatible with data published previously. Genetic variation may accompany these phenotypic differences. Anatolia, the mainland of Turkey, has received many migrations from the east as well as the west, and thus the population may present differences from the European group.

The G→A −238 and G→A −308 polymorphisms in the promoter region of the *TNFA* gene were not different from those of controls. Thus, these polymorphisms were not a defining factor for the development of disease. The genotype distributions were not significantly associated with a particular category in the Turkish group. However, the low numbers in some groups was an important drawback with respect to statistical significance.

It was of interest that the allele frequencies for both polymorphisms in the control Turkish population were different from those in the Czech control population. We have also found a difference between these two control populations in genotype distribution for the VNTR (variable number tandem repeat) polymorphism of the IL-1 receptor antagonist [16].

An important conclusion was that the G→A −308 polymorphism of the *TNFA* gene was associated with a poor prognosis in JIA in the Turkish group. The association may be due to the high level of TNF-α production associated with this particular allele. Indeed, high TNF levels have been associated with more severe disease in rheumatoid arthritis [8, 9]. On the other hand, this polymorphism may be in linkage disequilibrium with other severity gene(s). Thus, this polymorphism may be one of the many genetic factors affecting disease outcome in this cohort. However, this association was not present in the Czech group. This might be due to the fact that other factors have overcome the effect of this polymorphism in this group. As mentioned above, the Turkish group differs from the European group in certain demographic features and its low frequency of ANA, and different genetic risk factors may be operative. On the other hand, because the Czech patient group was larger, it may be possible that the association in the Turkish group may be associated with the low numbers of individuals studied. Further studies will clarify the significance of this association. We now intend to collect more cases on a multicentre basis.

Acknowledgements

This study was supported by the Scientific and Technical Research Council of Turkey (project SBAG-AYD-285). The work in the Czech Republic was supported by the Czech Ministry of Health (project CEZ MZ 00000023728).

References

1. Cassidy JT, Petty RE. Juvenile rheumatoid arthritis. In: Cassidy JT, Petty RE, eds. Textbook of pediatric rheumatology, 3rd edn. Philadelphia: W. B. Saunders, 1995:133–223.
2. Crawley E, Kay R, Sillibourne J, Patel P, Hutchinson I, Woo P. Polymorphic haplotypes of the IL-10 flanking region determine variable IL-10 transcription and are associated with particular phenotypes of JRA. Arthritis Rheum 1999;42:1101–8.
3. McDowell TL, Symons JA, Ploski R, Forre O, Duff GW. A genetic association between JRA and a novel IL-1α polymorphism. Arthritis Rheum 1995;28:221–8.
4. David J, Davies U, Rooney M, diGiovine F, Symons J, Woo P. Cytokine measurement in JCA [abstract]. Arthritis Rheum 1990;33(Suppl. 9):S118.
5. Rudwaleit M, Siegert S, Yin Z *et al*. Low T cell production of TNFα and IFN in ankylosing spondylitis: its relation to HLA B27 and influence of the TNFα-308 gene polymorphism. Ann Rheum Dis 2001;60:36–42.
6. Knight JC, Kwiatkowski D. Inherited variability of tumor necrosis factor production and susceptibility to infectious disease. Proc Assoc Am Phys 1999;111:290–8.
7. Wilson AG, Symons JA, McDowell TL *et al*. Effects of a polymorphism in the human TNF alpha promoter on transcriptional activation. Proc Natl Acad Sci USA 1997;94:3195–9.
8. Martinez A, Fernandez-Arquero M, Pascual-Salcedo D *et al*. Primary association of TNF region genetic markers with susceptibility to rheumatoid arthritis. Arthritis Rheum 2000;43:1366–70.
9. Verweij CL. TNF gene polymorphisms as severity markers in RA. Ann Rheum Dis 1999;58(Suppl. 1):120–6.
10. McCurdy D. Genetic susceptibility to the connective tissue diseases. Curr Opin Rheumatol 1999;11:399–407.
11. Rood MJ, van Krugten MV, Zanelli E *et al*. TNF-308A and HLA-DR3 alleles contribute independently to susceptibility to systemic lupus erythematosus. Arthritis Rheum 2000;43:129–34.
12. Petty RE, Southwood TR, Baum J *et al*. Revision of proposed classification criteria for juvenile idiopathic arthritis: Durban 1997. J Rheumatol 1998;25:1991–5.
13. Kosinski M, Zhao SZ, Dedhiya S, Osterhaus JT, Ware JE. Determining minimally important changes in generic and disease specific health related quality of life questionnaires

in clinical trials of rheumatoid arthritis. Arthritis Rheum 2000;43:1478–87.
14. Ozdogan H, Kasapcopur O, Dede H *et al*. JCA in a Turkish population. Clin Exp Rheumatol 1991;9:431–5.
15. Ozen S, Karaaslan Y, Ozdemir O, Saatci U, Bakkaloglu A, Tezcan S. Prevalence of juvenile chronic arthritis and familial Mediterranean fever in Turkey: a field study. J Rheumatol 1998;25:2445–9.
16. Vencovsky J, Jarosova K, Ruzickova S *et al*. Higher frequency of allele 2 of the interleukin-1 receptor antagonist gene in patients with juvenile idiopathic arthritis. Arthritis Rheum 2001;44:2387–91.